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Do very small adipocytes in subcutaneous adipose tissue (a proposed risk factor for insulin insensitivity) have a fetal origin?

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SUMMARY

Background & aims: Previous studies have shown that fetal life malnutrition affects preferences for fat deposition in the body thereby predisposing for visceral adipocity and associated disorders in glucose-insulin regulation. In this study, we aimed to test the hypotheses that late-gestation undernutrition 1) has long-term differential impacts on development, expandability and metabolic features in subcutaneous as compared to perirenal and mesenteric adipose tissues, which 2) will predispose for visceral obesity upon exposure to an obesogenic diet in early postnatal life.
Methods: Twin-bearing last trimester ewes received diets supplying 100% (NORM) or 50% (LOW) of protein and energy requirements. Lambs received moderate, low-fat (CONV) or high-carbohydrate-high-fat (HCHF) diets from 3-days until 6-months of age (just after puberty), and then half the lambs (including all males) were sacrificed. Remaining animals (exclusively females) received a low-fat, grass-based diet until sacrificed at 2-years of age (adulthood). In subcutaneous, perirenal and mesenteric fat, energy metabolism related gene expressions and fatty acid composition were

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determined. Histological evaluations were performed of subcutaneous and perirenal fat. The late-gestation undernutrition reduced whole-body insulin sensitivity and increased the risk of obesity-induced mesenteric adiposity in the sheep used in the experiment.
Results: A deviating morphology of subcutaneous adipose tissue with greater occurrence of very small adipocytes (<40 μm in diameter) and collagen infiltration was observed in the non-obese LOW/CONV lambs, and after dietary correction (and associated body fat loss) it became apparent in all adult LOW sheep. LOW lambs deposited more fat in visceral compared to subcutaneous fat when exposed to the obesogenic HCHF diet. Prenatal undernutrition had differential impacts in subcutaneous versus perirenal fat on expressions of glucose-insulin signaling and lipid metabolism genes and on fatty acid composition, but these prenatal impacts were not sustained into adulthood, except to a limited extent in perirenal fat, where C14:0 was decreased in LOW sheep.
Conclusions: The present study showed that greater preponderance of very small adipocytes, increased collagen infiltration and reduced subcutaneous lipid accumulation ability, as well as altered perirenal fat preferences for accumulation of C14:0 can have a fetal origin. Disturbance of normal (subcutaneous) adipose tissue development may play a key role in linking fetal malnutrition to disease risk later in life.

Abbreviations

ACTB	beta-actin
AMPK	AMP-activated kinase
CONV	moderate diet fed to lambs from 3 days to 6 months of age
FA	fatty acid
FAS	fatty acid synthase
FTO	fat mass and obesity associated protein
GLUT1 and GLUT4	glucose transporter 1 and 4
HCHF	high-carbohydrate-high-fat diet fed to lambs from 3 days to 6 months of age
INSRβ	insulin receptor beta subunit
IRS1	insulin receptor substrate 1
JNK	C-Jun N-terminal kinase
JSP1	JNK stimulatory phosphatase 1
LOW	50% maternal nutrition through late gestation
MESAT	mesenteric adipose tissue
MUFA	monounsaturated fatty acid
n-3 and n-6 PUFA	omega-3 and omega-6 polyunsaturated fatty acids
NORM	100% maternal nutrition through late gestation
PCA	principal component analyses
PFA	Paraformaldehyde
PPARα and PPARγ	peroxisome proliferator-activated receptor alpha and gamma
PRAT	perirenal adipose tissue
PUFA	polyunsaturated fatty acids
SUBAT	subcutaneous adipose tissue
UCP2	uncoupling protein 2
VEGF	vascular endothelial growth factor

1. Introduction

Visceral adipocity is a major risk factor predisposing for development of insulin resistance and dysregulation of peripheral glucose uptake, however preferences for deposition of fat in the body may develop without associated disorders in glucose-insulin regulation [1,2]. Previous studies have shown that visceral and ectopic fat deposition are influenced by the ability of the subcutaneous adipose tissue (SUBAT) to expand in situations with excess availability of nutrients, and a reduced subcutaneous expandability will increase the risk of lipid overflow and fat deposition in other adipose depots and in non-adipose tissues [3,4]. Restricted subcutaneous expandability has been linked to inability of adipocytes to differentiate properly [5], and interestingly, development of insulin resistance and adipose inflammation in equally obese human subjects appear to be associated with the presence of a distinct sub-population of very small adipocytes (<40 μm in diameter) [5,6]. Thus, adipose tissues are not only organs passively responding to stimuli implicated in disorders such as obesity and the metabolic syndrome [7,8], but organs with distinct developmental trajectories and regulatory functions, which can contribute to the development of obesity related disorders.

It has been known for some time that exposure to nutrient restriction in late gestation can predispose for glucose intolerance, insulin resistance and abdominal obesity later in life [9–11]. Individuals exposed to undernutrition in fetal life are often born small-for-gestational-age with an increased ratio of visceral-to-subcutaneous fat [12,13], and the reduced subcutaneous expandability can persist into adult life [14]. This suggests that fetal life history have differential implications for development and maturation in different adipose tissues by as yet unknown mechanisms, which in turn affects their metabolic functions and expandabilities later in life.

In this study, we aimed to test the hypotheses that late-gestation undernutrition 1) has long-term differential impacts on development, expandability and metabolic features in subcutaneous as compared to perirenal and mesenteric adipose tissues, which 2) will predispose for visceral obesity upon exposure to an obesogenic diet in postnatal life.

To test these hypotheses, we used our Copenhagen sheep model [14] to assess impacts of undernutrition during late gestation equivalent to the human third trimester. Adipose morphology, gene expression for markers of insulin signaling and energy metabolism, and fatty acid (FA) composition were studied in three adipose depots: mesenteric (MESAT), subcutaneous (SUBAT), and perirenal (PRAT) adipose tissues at 6-months (just after puberty) and 2-years (young adulthood) of age.

2. Materials and methods

Handling of experimental animals and all experimental procedures were approved by The Danish National Committee on Animal Experimentation and conducted in accordance with the EU Directive 2010/63/EU for animal experiments. The animal experiments were conducted at the experimental farm *Rørrendegård*, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark. This report adhere to the ARRIVE Guidelines for reporting of animal experiments [15].

2.1. Experimental animals and treatments

The detailed information about the experimental animals and treatments has been earlier reported [14]. In short, 21 twin-pregnant ewes were during the last six weeks of gestation (term = 147 days) fed either a diet fulfilling the daily requirements for both energy and protein (N = 10; NORM) or 50% of these requirements (N = 11; LOW). From 3-days to 6-months of age, twin-lambs were allocated to each their diet: conventional (CONV; hay supplemented by milk replacer from 3-days to 8-weeks of age; adjusted to achieve moderate daily growth rates of 250 g day-1) or high-carbohydrate-high-fat (HCHF; max. 1 kg popped maize day-1, max. 0.5 L 38% fat dairy cream, and max 2.0 L milk replacer day-1 until 8-weeks of age and reduced to 0.5 L day-1 from 8-weeks to 6-months of age). At 6-months of age (after puberty), half of the lambs were sacrificed (15 male lambs plus 3 females), where after remaining lambs (18 females) were managed

as one group, all receiving the same low-fat, non-obesogenic grass-based diet from 6 months until 2-years of age (young adulthood) and then sacrificed. This latter diet consisted of grass silage provided according to daily energy and protein requirements in the winter (October–April) and pasture in the summer time (May–September). This resulted in four experimental groups (numbers of animals studied at 6-months/2-years of age are given in parenthesis): NORM/CONV (N = 5/5), NORM/HCHF (N = 4/4), LOW/CONV (N = 5/4), and LOW/HCHF (N = 5/5). At sacrifice, SUBAT (above the longissimus dorsi muscle) and two metabolically active abdominal adipose tissues in sheep: MESAT and PRAT were quickly dissected out, and samples were snap-frozen in liquid nitrogen and stored at −80 °C for gene expression and FA composition analysis or transferred to 4% PFA solution for histological evaluations.

2.2. Histology

Fixation of adipose tissues in PFA, embedding in paraffin, mounting of tissue sections in DPX-mounting-media (WVR, Herlev, Denmark) and Van Gieson staining for collagen/connective tissue infiltration were performed as described previously [16]. Paraffin blocks for MESAT were impossible to cut, and therefore histological evaluations were performed only on PRAT and SUBAT. For each block, two consecutive sections from each of three different sites in the block were collected (in total six) and they were separated by >300 μm to ensure that a given cell could not be represented in different sections. Five photos were taken randomly from one section from each site (i.e. a total of 15 photos per tissue per animal) at 10× and 5× magnification in PRAT and SUBAT, respectively, due to differences in intensities of staining in tissues from particularly HCHF fed lambs. Tissue sections were unbiased assigned a grade for different characteristics evaluated by visual comparison with reference pictures. Cell size was graded by 3- and 5-point scales, respectively, in PRAT and SUBAT, which had different variability in cell size (Supplementary Table 1). Only pictures devoid of significant presence of non-adipose structures were used for this purpose. Cross-sectional-area was manually determined in randomly chosen adipocytes in the reference pictures by applying a 15-point transparent grid using ImageJ software [17]. Grades were also assigned to all pictures for occurrence of a sub-population of very small cells (0 = no very small cells, 5 = large occurrence of very small cells), and in SUBAT these were manually characterized to have a mean cross-sectional area <1250 μm^2, equivalent to a diameter of <40 μm in a sphere with such a cross-sectional area. The extent of collagen infiltration was graded in all pictures by a 4-point scale ranging from 5 to 70% collagen infiltration (Supplementary Fig. 1). Collagen infiltration in reference pictures was calculated as the proportion of hits on a given tissue structure relative to the total number of hits in the whole picture using a 28-points transparent grid, as described previously [18].

2.3. RNA extraction and quantitative real-time PCR (qPCR)

RNA extraction, cDNA synthesis, quantity and integrity of isolated RNA and qPCR were performed as described previously [19] using beta-actin (*ACTB*) as reference gene. Adipose tissue samples (~120 mg) were homogenized and used for RNA extraction. RNA samples were only used for cDNA synthesis when RIN values were ≥6.5.

Expression levels were determined by qPCR for target genes known [20] to be involved in adipose tissues in basal and insulin stimulated glucose uptake (glucose transporter 1, *GLUT1*; and glucose transporter 4, *GLUT4*; respectively), insulin signaling (insulin receptor subunit beta; *INSRβ*; insulin receptor substrate 1, *IRS1*), lipid metabolism (lipogenesis: FA synthase, *FAS*; lipolysis: peroxisome proliferator-activated receptor alpha, *PPARα*), preadipocyte differentiation (peroxisome proliferator-activated receptor gamma, *PPARγ*), mitochondrial-derived reactive oxygen species control (uncoupling protein 2, *UCP2*) and angiogenesis (vascular endothelial growth factor, *VEGF*), leptin synthesis (leptin), as well as fat mass and obesity-associated protein (*FTO*). For each gene, a six-point standard curve was made, and efficiencies ($=10^{-1/(\text{slope of standard curve})}$) of primers were between 1.8 and 2.1 (which is equal to an increase of 80% and 110% of target nucleic acid in each amplification cycle, respectively) (Supplementary Table 2). All

coefficients of determination were ≥0.99. The original source of primer sequences have previously been published [18,19], except for *UCP2* and *leptin*, which were designed, and primer product size was confirmed by gel electrophoresis and PCR products sequenced to confirm targets.

2.4. Lipid analysis

The total lipid fraction was extracted from tissue samples by the Folch procedure [21], but with added water to compensate for the low water content of adipose tissues. The procedures for lipid extraction, FA trans-methylation and subsequent characterization by gas–liquid chromatography flame ionization detection were conducted as previously described [19].

2.5. Statistical analysis

Statistical analyses of qPCR and adipose histology data were performed using the SAS software ver. 9.2 (SAS Institute, Cary, NC, USA). Data for qPCR were first log transformed to fit normal distribution and then analyzed by a PROC Mixed model including prenatal diet, postnatal diet, age and their interactions as fixed effects and interaction of dam and lamb as random effect. Data for grading of adipocyte size and collagen infiltration were analyzed by one-way ANOVA on the four different experimental groups for 6-months old lambs and 2-years old sheep separately using the PROC NPAR1WAY procedure based on the distribution of Wilcoxon scores, and the Bonferonni correction was applied for multiple comparisons. Data for lipid contents were analyzed by two-way ANOVA with Prism 5 software (GraphPad Software, San Diego, CA, USA), using pre- and postnatal diet as independent factors. Data for lipid FA composition were also analyzed by principal component analysis (PCA) using the version 2.15.2 of the R software (R Foundation for Statistical Computing) as reported previously [19], and graphs were generated from Prism 5 software (GraphPad Software, San Diego, CA, USA).

3. Results

It should be noted that the impacts of prenatal and early-postnatal nutrition on adipose tissues characteristics at 6-months of age can be evaluated for males only, and at 2-years of age only for females, since all males had to be sacrificed at 6-months of age due to practical constraints. However, metabolic and endocrine responses during glucose, insulin and fasting tolerance tests were evaluated as previously reported [16,22] in all female and male lambs at 6-months of age, where gender effects were rare and mainly associated with fat deposition patterns [14]. Age-related changes for the mentioned traits have been evaluated in the female sheep studied at both 6-months and 2-years of age [16,22]. These previously published results will be referred to in the discussion when relevant to put the present findings into perspective.

As previously reported by Nielsen et al. [14], the HCHF compared to CONV diet induced extensive fat deposition in lambs (but not 2 year old female sheep) in MESAT (278 vs. 1803 g ~3.88 and 2.07% of body weight, respectively) and PRAT (123 vs. 1129 g ~0.31 vs. 2.42% of body weight, respectively) and thicker dorsal SUBAT layer (1.60 vs. 0.96 mm) ($P < 0.001$ for all). CT scans of females showed that LOW compared to NORM lambs and adult sheep had thinner dorsal SUBAT layer (1.60 vs. 13.17 mm), and LOW-HCHF female lambs had higher ratios of visceral:subcutaneous fat (0.69) compared to NORM-HCHF (0.49) and CONV fed (appr. 0.1) ($P < 0.01$).

3.1. Adipose tissue histology

Six-months old (adolescent) lambs: The postnatal obesogenic HCHF as compared to CONV diet expectedly induced a massive increase in adipocyte cell size in SUBAT ($P = 0.01$; Fig. 1A: panels to the right) and PRAT ($P = 0.0037$; Supplementary Fig. 2A: panels to the right). SUBAT from HCHF fed lambs (Fig. 1A: panels to the right) as well as from NORM/CONV lambs (Fig. 1A: top left) contained adipocytes of quite uniform size and with a characteristic angular shape and with little non-adipocyte structures

(particularly in HCHF fed lambs). SUBAT from LOW/CONV lambs (Fig. 1A: bottom left) had more extensive presence of collagen and non-collagen extracellular material compared to the other groups ($P = 0.02$; $P = 0.038$; $P = 0.085$ vs. NORM/HCHF, LOW/HCHF and NORM/CONV, respectively) with cell shapes of more irregular appearance and greater numbers of very small adipocytes (<1250 μm^2 equivalent to <40 μm in diameter in a sphere with similar cross-sectional area). All PRAT pictures taken in lambs as well as in adult sheep contained very little extracellular material and was devoid of very small adipocytes irrespectively of the pre- or postnatal nutrition exposure (Supplementary Fig. 2).

Two-years old (adult) sheep: The deviating SUBAT morphology of LOW/CONV lambs (higher proportion of extracellular matrix including collagen and a more numerous population of very small cells) was observable also in adulthood (Fig. 1B top left), and interestingly became evident also in the LOW/HCHF adults (Fig. 1B top right) after they had been fed a moderate diet for 1½ years and normalized their body fat content [14]. There were no differences in the overall adipocyte size grading between the groups, but LOW/CONV had more collagen infiltration compared to NORM/CONV sheep ($P = 0.038$) (not significant compared to LOW/HCHF). Maximum scores for collagen infiltration and occurrence of very small cells were only assigned to SUBAT from LOW sheep (in 4 out of the 9 sheep). No NORM sheep scored higher than 2 for collagen infiltration.

3.2. Adipose tissue gene expressions

Expressions of all the studied genes were affected by the prenatal nutrition history, but in a tissue and age-specific way.

Six-months old (adolescent) lambs: Prenatal nutrition impacts were observed exclusively in SUBAT (Fig. 2). The non-obese LOW/CONV lambs had higher mRNA expressions in SUBAT for *IRS1*, *FAS*, *FTO* (Fig. 2 panels b, d and f, respectively, all $P < 0.05$) compared to the other three groups and of *PPARα* compared to LOW/HCHF lambs ($P < 0.05$, Fig. 2c). *UCP2* expression on the other hand was reduced in both LOW groups compared to NORM/CONV ($P < 0.05$, Fig. 2e).

The postnatal diet affected mRNA expressions in all three adipose tissues, but MESAT was the most responsive. Thus in HCHF lambs, MESAT mRNA expressions were reduced for *INSRβ*, *IRS1*, *GLUT4*, *PPARα*, *PPARγ*, *FAS*, *FTO* and *VEGF* (all $P < 0.05$, Supplementary Fig. 3). In SUBAT, the HCHF diet reduced mRNA expressions for *GLUT4* ($P < 0.05$, data not shown), *IRS1* ($P < 0.01$, Fig. 2b), and *FAS* ($P < 0.01$, Fig. 2d), whereas expression was increased for *leptin* ($P < 0.05$, data not shown). In PRAT, the HCHF lambs had reduced mRNA expressions for *GLUT4* ($P < 0.001$, Fig. 3b) and increased expression of *leptin* ($P < 0.05$, data not shown) compared to CONV lambs.

Two-years old (adult) sheep: Fetally induced changes in gene expression emerged in PRAT and MESAT in the adult sheep, which were not observed in lambs. In PRAT, LOW sheep had increased *GLUT4* mRNA expression ($P < 0.01$, Fig. 3b), but reduced *FAS* expression (lower in both LOW groups compared to NORM/CONV sheep; $P < 0.05$, Fig. 3d). In MESAT, LOW sheep had increased mRNA expression of *PPARα*, and LOW/HCHF sheep had increased mRNA expressions of *GLUT1* and *VEGF* (all $P < 0.05$, Supplementary Fig. 3) compared to LOW/CONV sheep. In SUBAT, the fetal induced changes in gene expression observed in lambs were no longer apparent in the adult sheep, except for *FTO* mRNA expression, which was higher in LOW/HCHF compared to other sheep ($P < 0.05$, Fig. 2f), whereas the highest expression level among lambs was in the LOW/CONV group.

None of the effects of the HCHF diet observed in lambs could be found in the adult sheep, but others emerged. In PRAT, mRNA expression was increased for *GLUT4* in HCHF sheep (contrary to what was observed in lambs; $P < 0.05$, Fig. 3b), whereas *INSRβ* expression was reduced among NORM sheep previously fed the HCHF diet. In MESAT, *VEGF* and *GLUT4* mRNA expression was as mentioned increased ($P < 0.05$, Supplementary Fig. 3) in LOW/HCHF compared to LOW/CONV sheep (opposite to the depressive effect of the HCHF diet in lambs).

3.3. Fatty acid composition in adipose tissues

Six months old (adolescent) lambs: The FA compositions of adipose tissues were as expected strongly influenced by the postnatal diet (Fig. 4 and Supplementary Fig. 4). FA present in high concentrations in fats from maize (linoleic acid, C18:2 n-6) and dairy cream (saturated FAs with chain lengths from C10 to

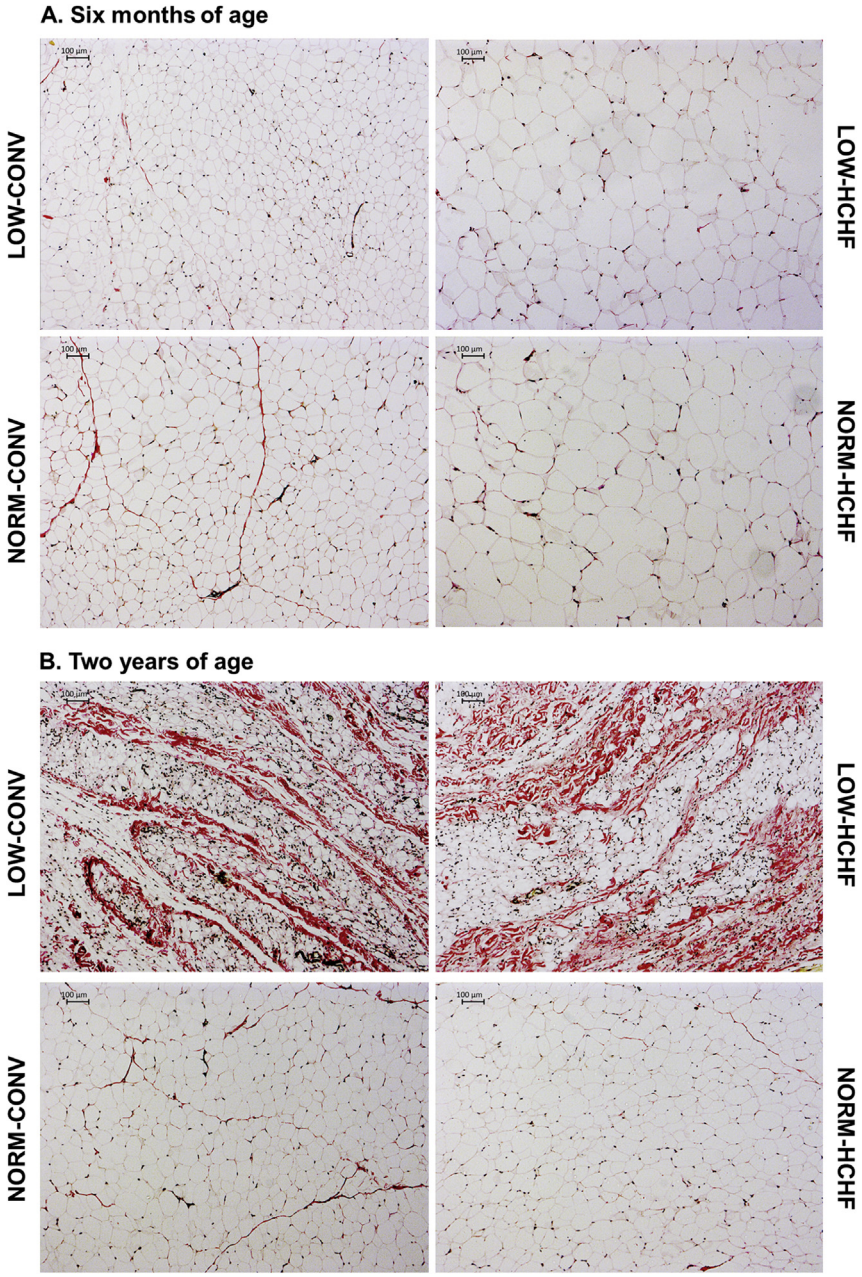

Fig. 1. Morphology of Van Gieson stained subcutaneous adipose tissue from 6-months old adolescent lambs and 2-years old adult sheep. Panel A: examples of pictures from the 4 groups of lambs, used to grade cell size (and with negligible collagen infiltration) showing a larger population of very small cells in the LOW/CONV (bottom left) relative to the other groups and extensive hypertrophy in adipocytes from HCHF lambs (pictures to the right). Panel B: morphological characteristics observed in slides from 4 out of 9 adult LOW sheep and not restricted to a specific early postnatal diet (pictures at the top) with extensive collagen infiltration (grade 4), which was never observed to the same extent among NORM sheep (max grade assigned = 2). NORM and LOW refer to prenatal nutrition during the last 6-weeks of gestation, where twin pregnant dams were fed diets fulfilling 100% of daily energy and protein requirements or 50% of those requirements. CONV and HCHF refer to moderate or obesogenic, high-carbohydrate-high-fat diets, respectively, fed to the lambs during the first 6 months of postnatal life.

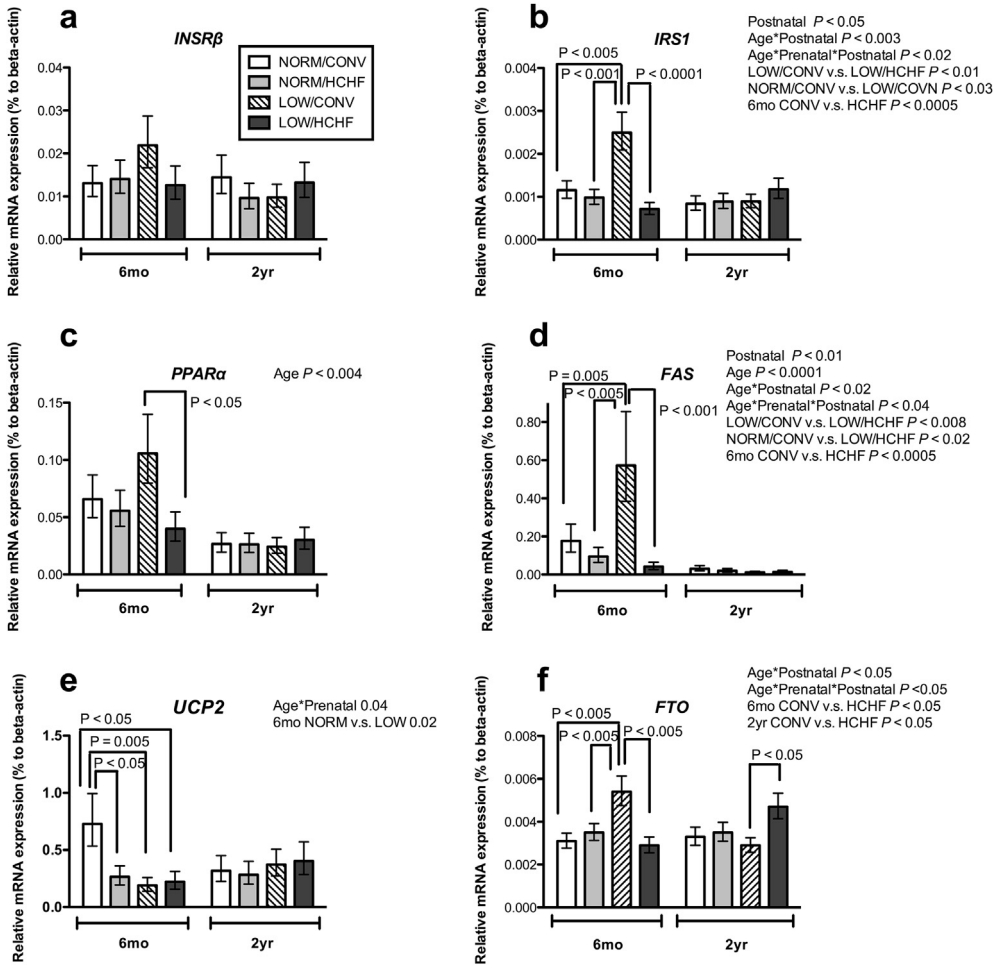

Fig. 2. Impact of nutrition in late gestation and early postnatal life on mRNA expression of target genes in subcutaneous adipose tissue in 6-months old adolescent lambs and 2-years old adult sheep. Panel a: insulin receptor beta (*INSRβ*), panel b: insulin receptor substrate 1 (*IRS1*), panel c: peroxisome proliferator-activated receptor alpha (*PPARα*), panel d: fatty acid synthase (*FAS*), panel e: the mitochondrial uncoupling protein 2 (UCP2), and panel f: fat mass and obesity-associated protein (*FTO*). Data are the ratios of expression relative to beta-actin for each target gene, and are expressed as least square mean ± standard error of the mean. NORM/CONV, NORM/HCHF, LOW/CONV, LOW/HCHF refer to experimental treatment groups. NORM and LOW refer to prenatal nutrition during the last 6-weeks of gestation, where twin pregnant dams were fed diets fulfilling 100% of daily energy and protein requirements or 50% of those requirements, respectively. CONV and HCHF refer to moderate and high-carbohydrate-high-fat diets, respectively, fed to the lambs during the first 6 months of postnatal life. Legends are shown at the top right corner of panel a.

C16) were enriched in adipose tissues from the HCHF lambs fed this diet ($P < 0.0001$, Fig. 4 and Supplementary Fig. 4). Despite higher intake of C17:0 from dairy fat, the HCHF compared to CONV lambs had substantially lower adipose concentrations of this and other odd-chained FAs ($P = 0.0001$, Fig. 4), which are also synthesized endogenously from propionic acid, derived from forestomach fermentation.

In MESAT and PRAT (Fig. 4a and b, respectively), linoleic acid concentration was higher in LOW than NORM lambs when fed the CONV diet, but lowest in LOW lambs when fed the HCHF diet ($P < 0.05$ for the pre- and postnatal dietary interaction), and n-6/n-3 polyunsaturated fatty acid ratios became lower in all adipose tissues in LOW/HCHF compared to NORM/HCHF lambs ($P < 0.005$ for the

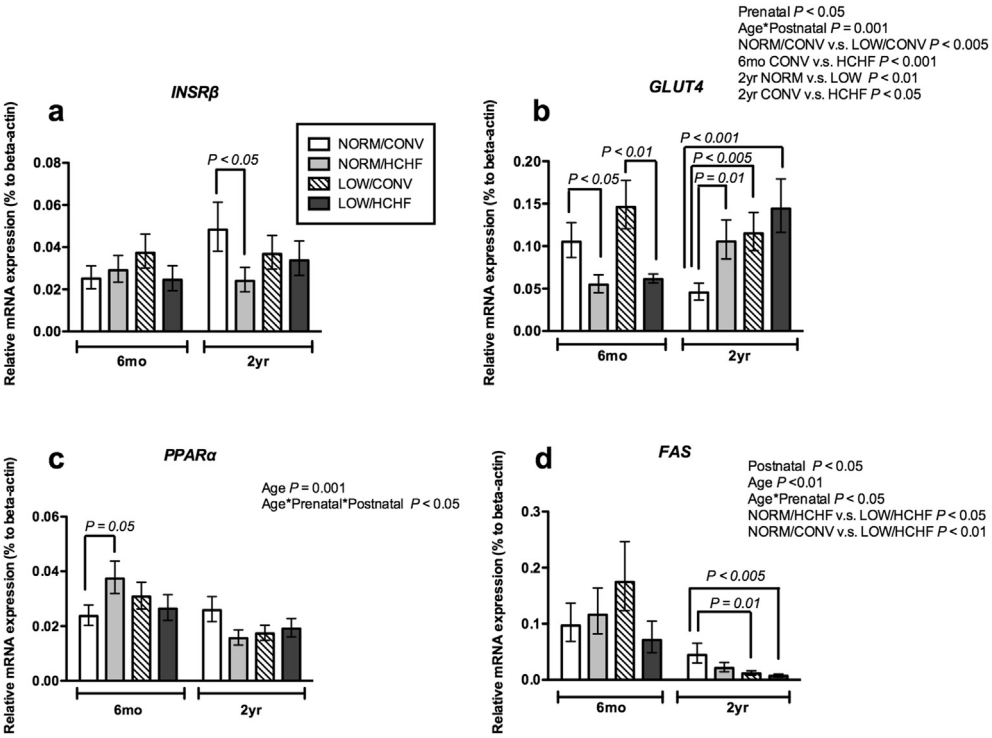

Fig. 3. Impact of nutrition in late gestation and early postnatal life on mRNA expression of target genes in perirenal adipose tissue in 6-months old adolescent lambs and 2-years old adult sheep. Panel a: insulin receptor beta (*INSRβ*), panel b: insulin-dependent glucose transporter 4 (GLUT4), panel c: peroxisome proliferator-activated receptor alpha (*PPARα*), and panel d: fatty acid synthase (*FAS*). Data (least square mean ± standard error of the mean) are the ratios of expression relative to beta-actin for each target gene. NORM/CONV, NORM/HCHF, LOW/CONV, LOW/HCHF refer to experimental treatment groups. NORM and LOW refer to prenatal nutrition during the last 6-weeks of gestation, where twin pregnant dams were fed diets fulfilling 100% of daily energy and protein requirements or 50% of those requirements, respectively. CONV and HCHF refer to moderate and high-carbohydrate-high-fat diets, respectively, fed to the lambs during the first 6 months of postnatal life. Legends are shown at the top right corner of panel a.

pre- and postnatal nutrition interaction, Fig. 5). Concentrations of medium-chained FAs (MCFA, C10–C14) tended to be increased in LOW lambs in MESAT ($P = 0.06$, Fig. 4b) irrespective of the postnatal diet.

Two years old (adult) sheep: Although all adult sheep had been fed the same low-fat diet for 1½ years, resulting in normalization of the body fat content of the HCHF sheep, FA composition (Fig. 6) in adipose tissues, as shown in PCA plots (Supplementary Fig. 5), continued to reflect the diet received in early postnatal life, but less pronounced than observed in the lambs. The FA patterns were very similar across the three depots, and long-term effects of the prenatal nutrition history were only found in PRAT, where LOW sheep had decreased contents of myristic acid ($P < 0.01$, Fig. 6) and increased C16:0/C18:0 ratio ($P < 0.05$, Fig. 5), which was not observed in lambs.

4. Discussion

4.1. Fetal origin of very small adipocytes in subcutaneous adipose tissue

We are to our knowledge the first to demonstrate that occurrence of a special subpopulation of very small adipocytes in SUBAT may have a fetal origin, but PRAT may not be similarly affected. Several human studies have shown that a high preponderance of very small adipocytes in SUBAT is associated

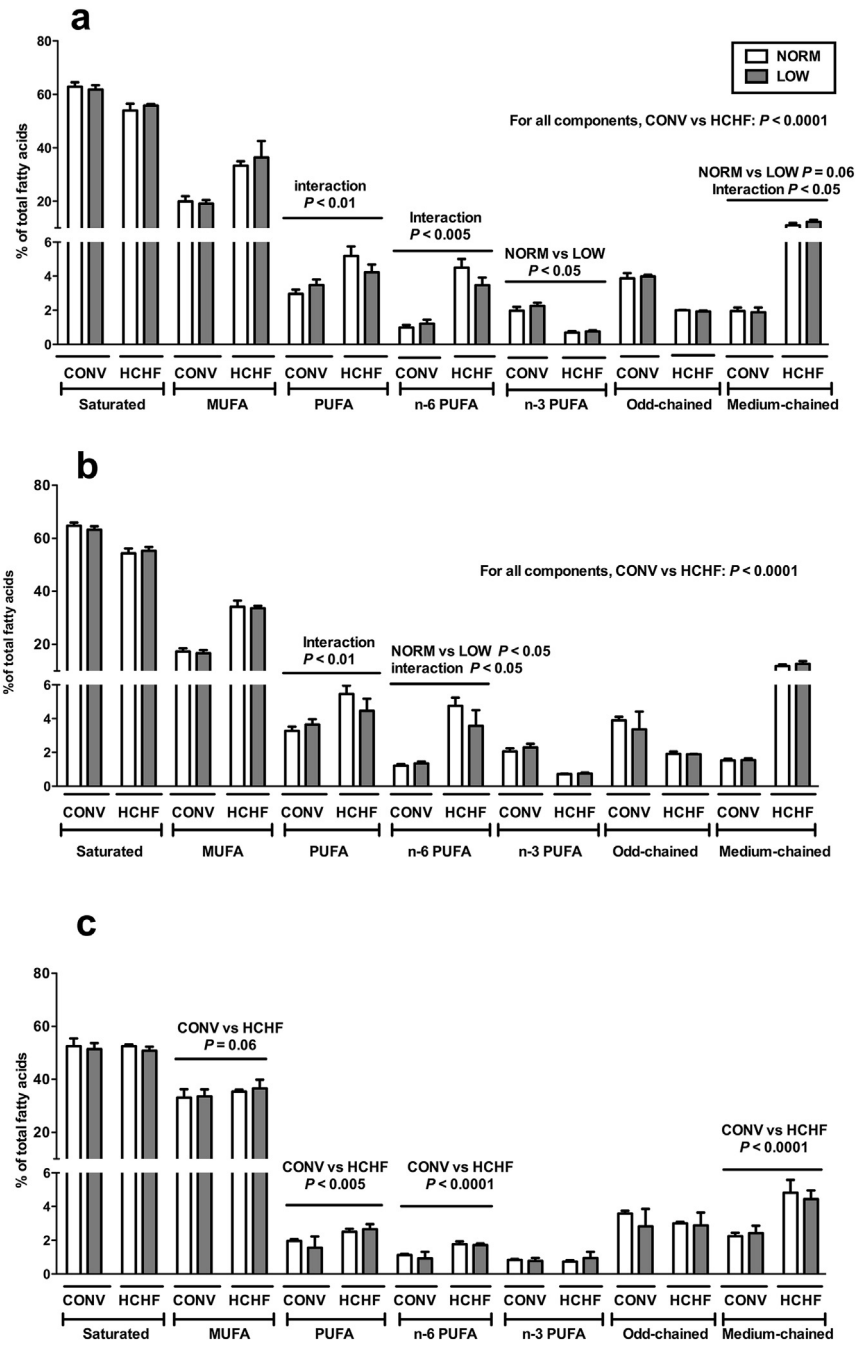

Fig. 4. Composition of total fatty acids (weight %) in adipose tissues of 6-months old adolescent lambs. Panel a, mesenteric fat; panel b, perirenal fat; and panel c, subcutaneous fat. Seven classes of fatty acids are shown: saturated fatty acids (Saturated), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-6 polyunsaturated fatty acids (n-6 PUFA), omega-3 polyunsaturated fatty acids (n-3 PUFA), odd chained fatty acids (Odd-chained), and medium chained fatty acids (6–12 carbon atoms, Medium-chained). Data are expressed as mean ± standard deviation. NORM and LOW refer to prenatal nutrition during the last 6-weeks of gestation, where twin pregnant dams were fed diets fulfilling 100% of daily energy and protein requirements or 50% of those requirements, respectively. CONV and HCHF refer to moderate and high-carbohydrate-high-fat diets, respectively, fed to the lambs during the first 6 months of postnatal life. Legends are shown on the top right of panel a. Experimental factors with P-values <0.05 are shown on top of the bars.

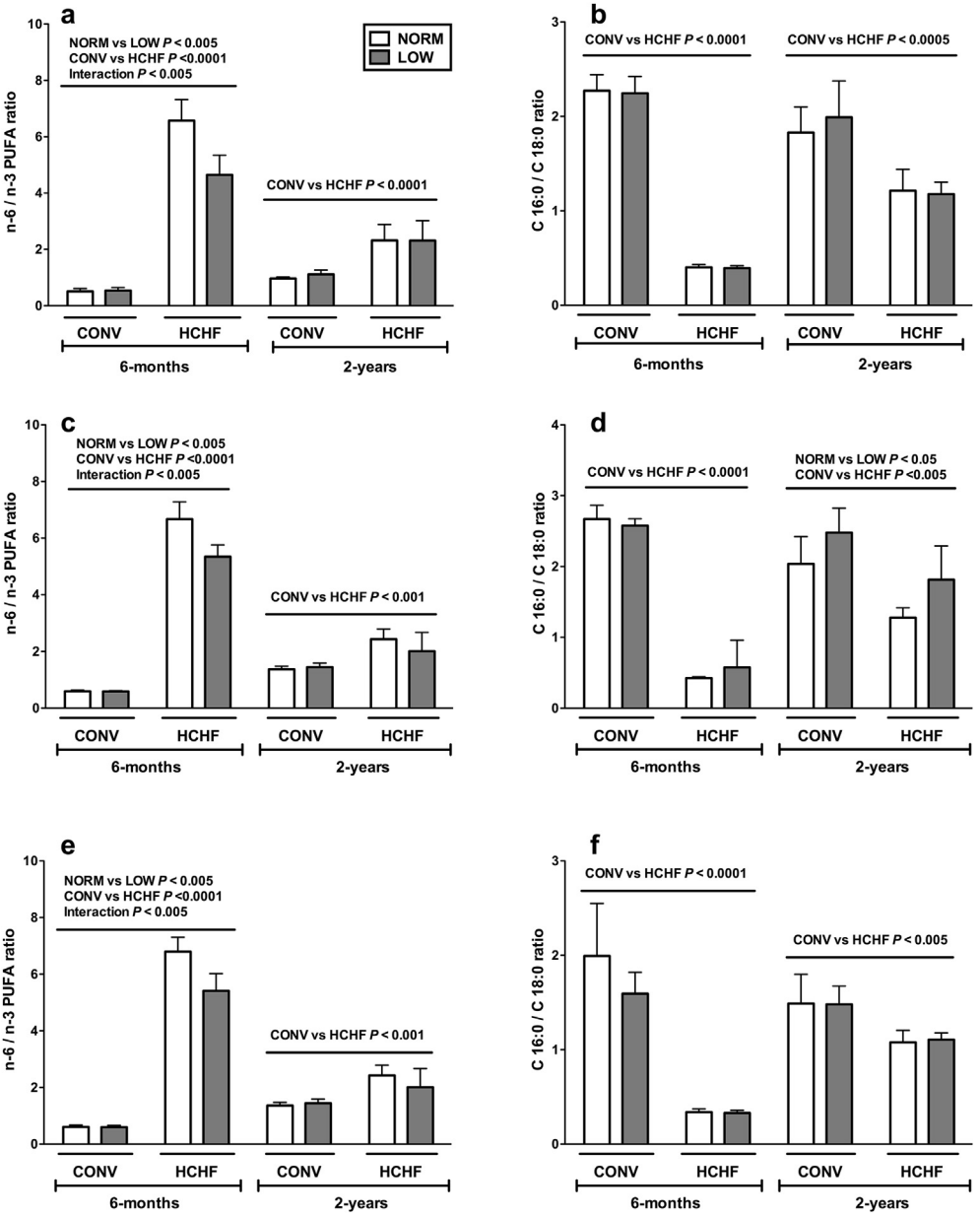

Fig. 5. Omega-6/omega-3 polyunsaturated fatty acid ratio (n-6/n-3 PUFA ratio) and C16:0/C18:0 fatty acid ratio in adipose tissues of 6-months old lambs and 2-years old sheep. Panel a and b, mesenteric fat; panel c and d, perirenal fat; and panel e and f, subcutaneous fat. Panel a, c, and e, n-6/n-3 PUFA ratio; and panel b, d, and f, C16:0/C18:0 ratio. Data are expressed as mean ± standard deviation. NORM and LOW refer to prenatal nutrition during the last 6-weeks of gestation, where twin pregnant dams were fed diets fulfilling 100% of daily energy and protein requirements or 50% of those requirements, respectively. CONV and HCHF refer to moderate and high-carbohydrate-high-fat diets, respectively, fed to the lambs during the first 6 months of postnatal life. Legends are shown on the top right of panel a. Experimental factors with P-values <0.05 are shown on top of the bars.

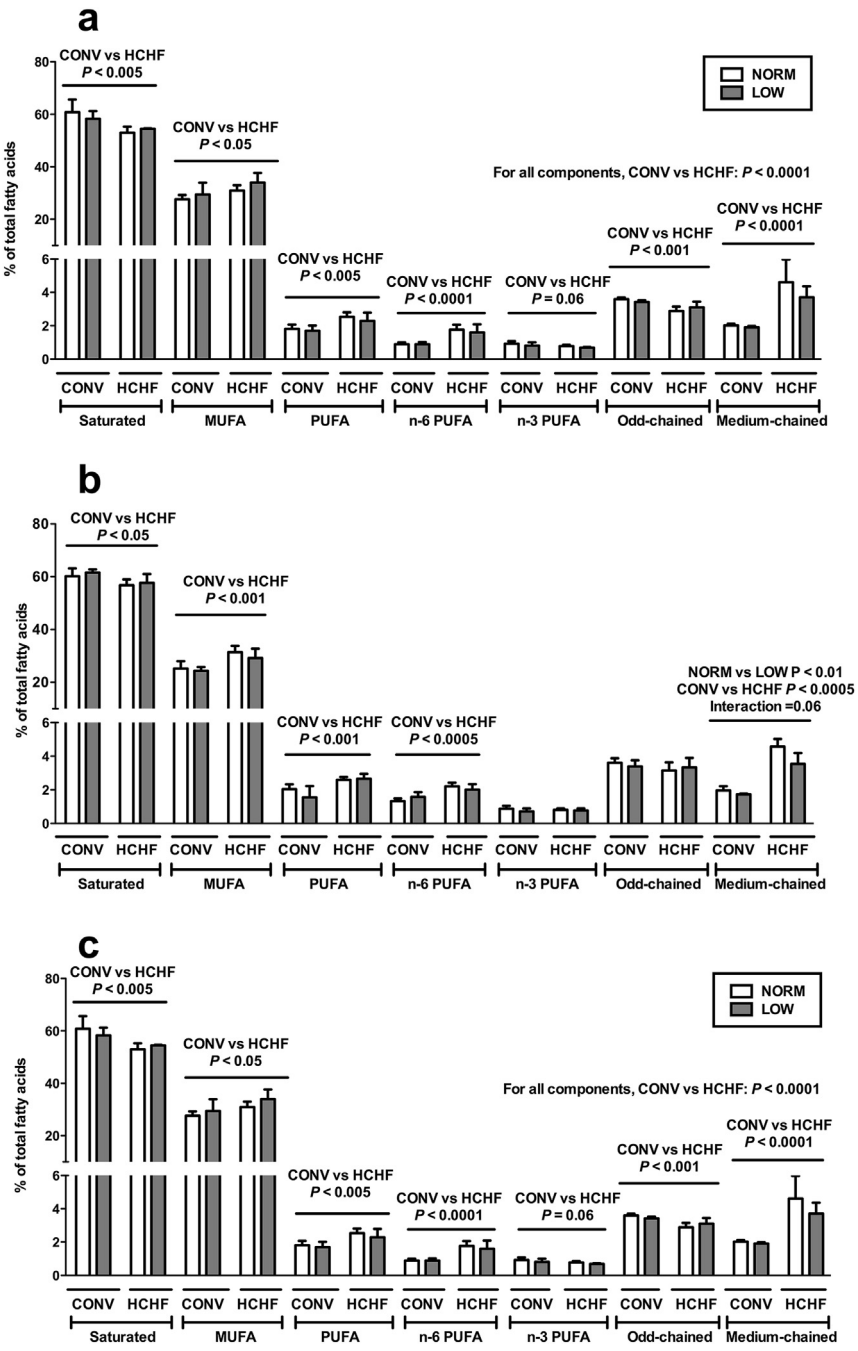

Fig. 6. Composition of total fatty acids (weight %) in adipose tissues of 2-years old sheep. Panel a, mesenteric fat; panel b, perirenal fat; and panel c, subcutaneous fat. Seven classes of fatty acids are shown: saturated fatty acids (Saturated), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-6 polyunsaturated fatty acids (n-6 PUFA), omega-3 polyunsaturated fatty acids (n-3 PUFA), odd chained fatty acids (Odd-chained), and medium chained fatty acids (6–12 carbon atoms, Medium-chained). Data are expressed as mean ± standard deviation. NORM and LOW refer to prenatal nutrition during the last 6-weeks of gestation, where twin pregnant dams were fed diets fulfilling 100% of daily energy and protein requirements or 50% of those requirements, respectively. CONV and HCHF refer to moderate and high-carbohydrate-high-fat diets, respectively, fed to the lambs during the first 6 months of postnatal life. Legends are shown on the top right of panel a. Experimental factors with P-values <0.05 are shown on top of the bars.

with increased degree of insulin resistance in equally obese subjects [6,23] and increased expression of inflammatory genes in SUBAT in healthy moderately obese humans [5]. In line with this, we have previously reported that insulin sensitivity was reduced in LOW lambs and sheep and glucose intolerance was reduced in LOW-HCHF lambs used in this experiment [16]. Very small cells isolated by collagenase digestion from SUBAT in the above-mentioned human studies had comparable diameters (<40 μm) to those observed in this study in LOW animals (<1250 μm^2 equivalent to <40 μm spherical cell diameter).

Fetal derived morphological changes in SUBAT, evident in both lambs and adult sheep, could not be linked to systematic changes in gene expression, which were only found in lambs. The increased SUBAT expression of genes involved in insulin signaling and energy storage in LOW/CONV compared to NORM/CONV lambs (Fig. 2) were not in line with the observed reduced SUBAT expandability and aforementioned whole body insulin sensitivity in all LOW animals. Such paradoxical up-regulation of insulin signaling molecules has, however, also been reported in skeletal muscle of insulin insensitive human subjects associated with hyperinsulinemia [24], and could reflect a compensatory upregulation in response to impaired insulin signaling distal to the insulin receptor, but this was clearly abolished during obesity development (Fig. 2), and did not persist into adulthood.

Gene expression patterns determined on the total adipose cell population, as in this study, may however not be representative of the SUBAT very small adipocytes [23,25]. Thus, isolated very small as compared to large adipocytes have in other studies with human SUBAT [23] and epididymal fat from obese Zucker rats [25] been found to have markedly reduced expressions of adipocyte differentiation markers and markedly increased expressions of pro-inflammatory markers, and lower expression of a marker, COL6A3, associated with insulin resistance [26].

Furthermore, reduced gene expressions in preadipocytes isolated from low birth weight men of *leptin* and late differentiation markers such as *PPARγ2* and *GLUT4* has been associated with a more immature preadipocyte appearance and reduced ability to proliferate and differentiate [27]. This is interesting, since extensive expansion of fat mass in SUBAT in humans [6] although initially relying on hyperplasia, it relies much more extensively on a massive increase in adipose cell numbers (hyperplasia) later on. Reduced hyperplasic ability could thus provide explanation for the reduced expandability of SUBAT in LOW animals in this experiment, since adipocyte size (apart from the subpopulation of very small adipocytes) were similar in obese LOW and NORM lambs fed the HCHF diet.

We therefore suggest that late gestation undernutrition can give rise to development of a SUBAT with greater preponderance of very small adipocytes and fibrosis, which renders the SUBAT unable to expand normally in a nutrient surplus situation. Very small SUBAT adipocytes cannot, therefore, be considered a sign of a healthy adipose tissue. Rather greater preponderance of such cells indicates a dysfunctional SUBAT with reduced expandability, which according to [4] in nutrient surplus situations will increase the risk of excessive fat storage in visceral and non-adipose tissues, such as hepatocytes, β-cells and cardiomyocytes, with associated detrimental metabolic consequences. Such reduced SUBAT expandability can explain the previously reported increased preference for fat deposition in MESAT compared to SUBAT during obesity development in the LOW lambs in our experiment [14], and the increased gene expressions observed in adult LOW sheep of GLUT4 in PRAT and of GLUT1, VEGF and PPARα in MESAT in LOW/HCHF compared to LOW/CONV sheep would contribute to increase this risk. In contrast to SUBAT, gene expression changes related to fetal nutrition in PRAT and MESAT were only observed in the adult sheep and not in lambs. Due to the experimental design, we cannot rule out that this may reflect gender differences, rather than changes in the timing of fetal manifestations in the adipose tissues or long-term impacts of the altered expandability of SUBAT.

Another interesting observation was that prenatal undernutrition reduced accumulation of myristic acid in PRAT with a tendency also in the other adipose tissues. The physiological role of myristic acid is not very well understood, but it has been suggested that tissue levels are rate-limiting for protein myristoylation, which is an important and ubiquitous process regulating activity of an estimated 0.5–3% of the human proteome [28]. These include proteins implicated in membrane targeting, protein–protein interactions, and a variety of signal transduction pathways. Thus, localization of the AMP-activated kinase (AMPK) to the plasma membrane, its interaction and activation by LKB1 [29], and

activation of an enzyme implicated in proinflammatory signaling, C-Jun N-terminal kinase (JNK) stimulatory protein phosphatase 1 (JSP1) [30], are all dependent on myristoylation. Altered myristic acid levels can therefore modify the tendency for adipose inflammation and associated development of a metabolically unhealthy obese phenotype [30]. Future studies are needed to elucidate whether fetal induced changes in adipose tissue myristic acid metabolism is linked to the pro-inflammatory properties observed in very small SUBAT adipocytes [5,25] and associated greater collagen infiltration as we also observed in our sheep study (Fig. 1).

4.2. Effects of an obesogenic diet in early postnatal life are partly reversible

As previously reported, the obesogenic HCHF diet induced metabolic and endocrine changes resembling the metabolic syndrome in humans, and these obesity induced changes observed in the female lambs were to a large extent reversible upon feeding a moderate diet for 1½ years [14,16]. This was also the case for the adipose tissue morphological and gene expression characteristics reported here, except for the increased expressions for *GLUT1* and *VEGF* in MESAT in adult LOW-HCHF compared to LOW-CONV sheep, which as mentioned above may be associated with the greater preference for fat deposition in MESAT over SUBAT in this group [14]. Thus, dietary intervention can be a potent instrument to correct damages induced by early postnatal obesity development in species born precocial such as sheep, but individuals subjected to late fetal undernutrition may be limited in their capacity to reverse such effects.

The major exception to this reversibility regards postnatal diet-induced changes in adipose FA composition. Our results indicate that adipose tissues of sheep have a very slow turnover of FAs, as we also previously observed in muscle [19].

In conclusion, the present study showed that greater preponderance of very small adipocytes, increased collagen infiltration and reduced lipid accumulation ability in SUBAT, as well as altered PRAT preferences for accumulation of myristic acid can have a fetal origin, which can increase the risk of obesity-induced mesenteric adiposity. Disturbance of normal (subcutaneous) adipose tissue development during fetal life may thus play a key role in linking fetal malnutrition to obesity-associated disease risks later in life.

Statement of authorship

M.O. Nielsen developed the animal model, was in charge of the animal experimentation, contributed with overall evaluation of data and writing of the manuscript. L. Hou participated in the animal experimentation, was the responsible for all gene expression analyses, conducted tissue stainings, evaluated data and contributed with writing of the manuscript. L. Johnsen participated in the planning and conduct of the animal experiment, was responsible for the histological evaluation of tissues, evaluation of data and contributed with writing of the manuscript. P. Khanal participated in analyses of histological preparations, evaluation of the associated results, and contributed with writing of the manuscript. C.L. Bechshøft participated in the lipid analyses, and contributed with the interpretation of these data and contributed with writing of the manuscript. A.H. Kongsted participated in the planning and conduct of the animal experiment, provided the protocol for tissue stainings and supervised histological procedures, and participated in writing of the manuscript. A. Vaag participated to the experimental design and evaluation of data. L.I. Hellgren conducted lipid analyses, interpreted results and contributed with writing of the manuscript.

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Conflict of interest

No conflicts of interest are declared by the authors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yclnex.2016.05.003.

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